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ABSTRACT

Between the 16th and 18th centuries, *Aedes aegypti* (Diptera: Culicidae), a mosquito native to Africa, invaded the Americas, where it was successively responsible for the emergence of yellow fever (YF) and dengue (DEN). The species was eradicated from numerous American countries in the mid-20th century, but re-invaded them in the 1970s and 1980s. Little is known about the precise identities of *Ae. aegypti* populations which successively thrived in South America, or their relation with the epidemiological changes in patterns of YF and DEN. We examined these questions in Bolivia, where *Ae. aegypti*, eradicated in 1943, re-appeared in the 1980s.

We assessed the genetic variability and population genetics of *Ae. aegypti* samples in order to deduce their genetic structure and likely geographic origin. Using a 21-population set covering Bolivia, we analyzed the polymorphism at nine microsatellite loci and in two mitochondrial DNA regions (COI and ND4). Microsatellite markers revealed a significant genetic structure among geographic populations ($F_{ST} = 0.0627$, $P < 0.0001$) in relation with the recent re-expansion of *Ae. aegypti* in Bolivia. Analysis of mtDNA sequences revealed the existence of two genetic lineages, one dominant lineage recovered throughout Bolivia, and the second restricted to rural localities in South Bolivia. Phylogenetic analysis indicated that this minority lineage was related to West African *Ae. aegypti* specimens. In conclusion, our results suggested a temporal succession of *Ae. aegypti* populations in Bolivia, that potentially impacted the epidemiology of dengue and yellow fever.

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1. Introduction

Aedes (Stegomyia) aegypti, a mosquito native to Africa, was introduced throughout the New World via triangular transatlantic trade which occurred from 16th to 18th centuries (Soper, 1977). Colonization of Americas from the south USA to Argentina was followed by the emergence of devastating urban yellow fever virus (YFV, *Flaviviridae*) outbreaks from the mid-17th to the early 20th century (Nogueira, 2009). Although YFV was considered the worst *Aedes aegypti*-borne epidemic disease in tropical America at that time, dengue epidemics also occurred periodically throughout the region (Gubler, 2004). As a result of the *Ae. aegypti* eradication program initiated in the 1940s by the Pan American Health Organization (PAHO), the range of the species decreased significantly, and it was considered eradicated in numerous American countries, including Bolivia as early as 1943 (Tonn et al., 1982).

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Unfortunately, due to the break in vector control programs in the 1960s, *Ae. aegypti* progressively re-expanded in all countries where it was previously thought eradicated (Schatzmayr, 2000). The progressive reinvasion of *Ae. aegypti* was accompanied by the emergence of dengue as a major public health concern in the New World, and this was related to the appearance of the Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF/DSS) in 1981 (Vasilakis and Weaver, 2008).

In Bolivia, *Ae. aegypti* was recorded again in 1980 at Santa Cruz de la Sierra and two nearby localities (Pan American Health Organization, 1980) before becoming re-established throughout the country. The Santa Cruz department was affected in 1986–87 by a DEN-1 virus epidemic with about 7000 cases (Pan American Health Organization, 1994), and later in 1997 by a new epidemic wave involving a DEN-2 virus (Van der Stuyft et al., 1998). Since then, four well-documented outbreaks have occurred in Santa Cruz (Roca et al., 2009), where several serotypes co-existed. Surprisingly, and as observed throughout tropical America (Bryant et al., 2007), the YF virus remained restricted to its jungle cycle, and no epidemics recurred in the urban environment of Bolivia. Six YF

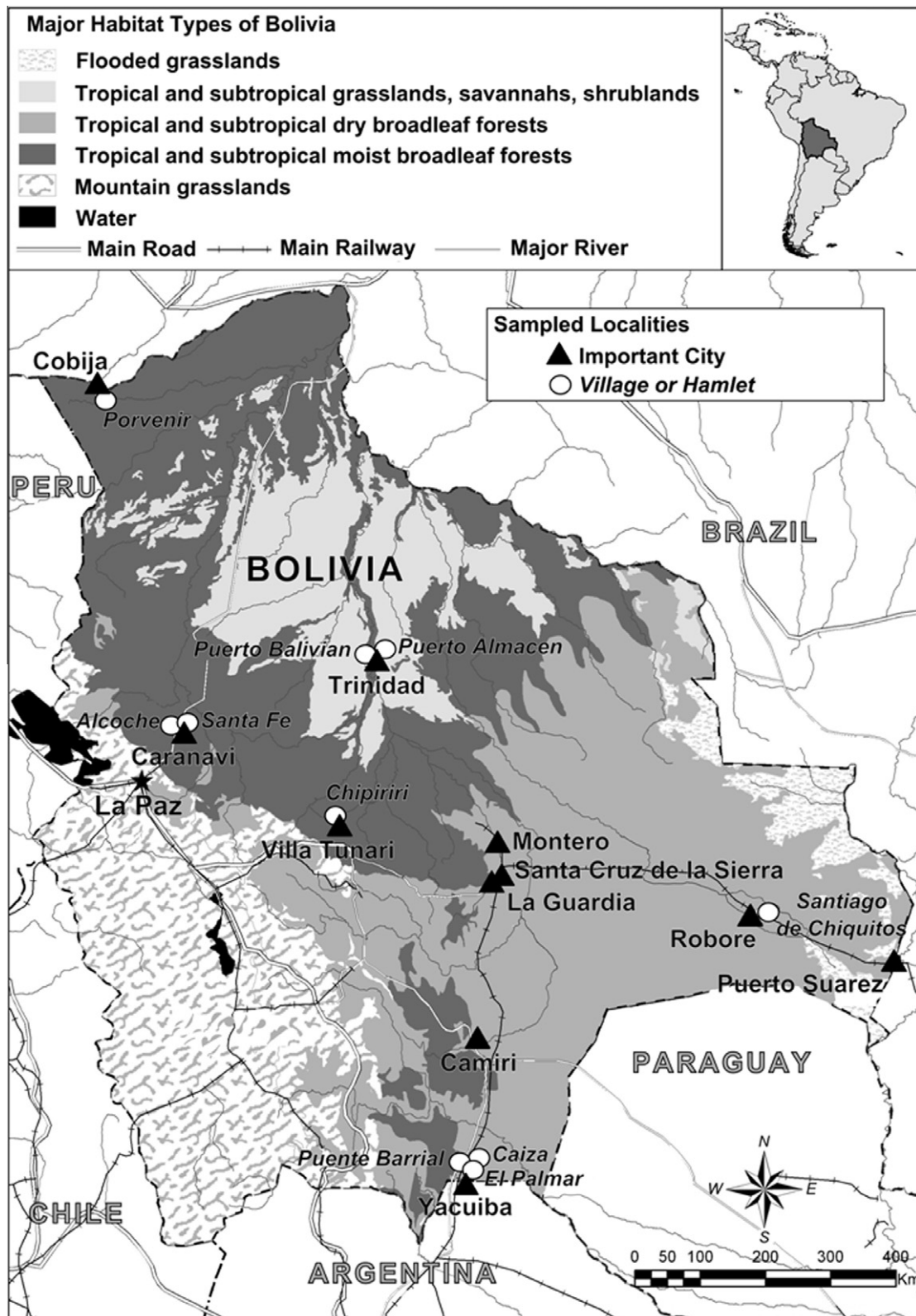


Fig. 1. Map showing the 21 localities and the major habitats sampled in 2005 and 2007 in Bolivia.

cases were reported in Santa Cruz de la Sierra in 1999 (Van der Stuyft et al., 1999), but while the *Ae. aegypti* population was found orally susceptible to YFV in the city (Mutebi et al., 2004), there was no evidence to incriminate this species in inter-human transmission, and other potential vectors such as *Haemagogus* sp. were also present at transmission sites. The reason for the non-resurgence of

epidemic YF is unclear, as little is known of temporal population genetic changes in *Ae. aegypti*, and subsequent differences in biological or vector competence. One possible explanation is the high YF 17D vaccine coverage in South America, particularly in urban areas, with a vaccine developed in the 1930s (Barrett and Monath, 2003).

Table 1
Ae. aegypti populations sampled in Bolivia, 2005–2007.

Locality	Date	Habitat	Stage	Geographic coordinates
Cobija ^{a,b}	Sep./2006	Urban	Larvae–Pupae/Adults	11°01'15"S; 68°45'49"W
Porvenir ^a	Sep./2006	Rural	Larvae–Pupae	11°14'07"S; 68°41'17"W
Trinidad ^{a,b}	Nov./2006	Urban	Larvae–Pupae	14°49'59"S; 64°53'59"W
Puerto Boliviano ^b	Nov./2006	Rural	Larvae–Pupae	14°47'37"S; 64°58'19"W
Puerto Almacen ^b	Nov./2006	Rural	Larvae–Pupae	14°52'17"S; 64°58'01"W
Alcoche ^{a,b}	Aug./2007	Rural	Larvae–Pupae	15°45'04"S; 67°40'29"W
Caranavi ^{a,b}	Aug./2007	Urban	Larvae–Pupae	15°50'09"S; 67°33'54"W
Santa Fe ^{a,b}	Aug./2007	Rural	Larvae–Pupae	15°48'30"S; 67°37'26"W
Villa Tunari ^{a,b}	Nov./2005	Urban	Larvae–Pupae	16°58'23"S; 65°25'02"W
Chipiriri ^b	Nov./2005	Rural	Larvae–Pupae	17°00'50"S; 65°27'22"W
Montero ^a	Jun./2006	Urban	Larvae–Pupae	17°20'32"S; 63°15'21"W
Santa Cruz de la Sierra ^{a,b}	April–May/2007	Urban	Larvae–Pupae	17°47'01"S; 63°10'56"W
La Guardia ^a	Nov./2007	Urban	Larvae–Pupae	17°53'34"S; 63°19'41"W
Robore ^{a,b}	Mar./2006	Urban	Larvae–Pupae	18°19'51"S; 59°45'23"W
Santiago de Chiquitos ^b	Mar./2006	Rural	Larvae–Pupae	18°20'23"S; 59°35'55"W
Puerto Suarez ^{a,b}	Mar./2006	Urban	Larvae–Pupae	18°58'01"S; 57°47'53"W
Camiri ^{a,b}	Jan./2007	Urban	Larvae–Pupae	20°02'20"S; 63°31'01"W
Yacuiba ^{a,b}	Jan./2006	Urban	Larvae–Pupae	22°01'03"S; 63°40'51"W
Puente Barrial ^b	Apr./2006	Rural	Larvae–Pupae	21°46'09"S; 63°34'02"W
Caiza ^{a,b}	Apr./2006	Rural	Larvae–Pupae/Adults	21°47'58"S; 63°33'24"W
El Palmar ^b	Aug./2007	Rural	Larvae–Pupae/Adults	21°52'04"S; 63°36'53"W

^a Samples analyzed using microsatellites.

^b Samples analyzed using mtDNA markers.

For about four centuries, the natural history of *Ae. aegypti* in the Americas was marked by major demographic disturbances (i.e. introduction, migration, extinction/re-colonization) that profoundly shaped the diversity and genetic structure of this invasive species at both the continental and national scales. The geographical origin of *Ae. aegypti* populations in the Americas remains unknown. Indeed, while it is commonly accepted that pioneer populations that initially invaded the New World originated in Africa (Urdaneta-Marquez and Failloux, 2010), it is also thought that later introductions in various American locations probably involved multiple geographical population sources. The existence of several differentiated genetic clusters across North-, Central- and South-American *Ae. aegypti* populations was suggested by a allozyme-based study (Tabachnick and Powell, 1979). Several more recent studies based on the use of mitochondrial DNA markers indicated the presence of two genetic lineages in Mexico, Central America (Gorochotegui-Escalante et al., 2002) and several South American countries, including Brazil (Scarpassa et al., 2008), Peru (da Costa-da-Silva et al., 2005) and Venezuela (Herrera et al., 2006). In Brazil, Scarpassa et al. (2008) demonstrated that one of the two lineages was related to East Africa and the other to West Africa. As vector competence for yellow fever and dengue viruses has been found to vary in populations of different origins (Tabachnick et al., 1985; Failloux et al., 2002), it is important to determine the source of colonizing populations.

In the past 30 years the genetic structure of *Ae. aegypti* populations has been assessed in several South American countries, such as Argentina, Brazil, Colombia, French Guyana, Peru and Venezuela, using a wide spectrum of genetic markers ranging from allozymes to DNA markers (nuclear and mtDNA) (see Urdaneta-Marquez and Failloux, 2010 for review). Overall, these studies showed that successive *Ae. aegypti* control programs implemented to fight yellow fever and dengue in South America had major and recurrent demographic effects that led to intense genetic drift and a high level of population genetic structure at the continental and local scales. Additionally, the *Ae. aegypti* population structure is exacerbated by the weak dispersion of females, that does not exceed a few hundred meters during their lifetime (Honório et al., 2003; Reiter et al., 1995). The main mode of long-distance dispersion is passive transportation.

Despite its central role in the transmission and persistence (i.e. vertical transmission) of dengue virus in Santa Cruz de la Sierra

(Le Goff et al., 2011), few entomological studies have been undertaken in Bolivia during the last 30 years. We therefore conducted a genetic survey of *Ae. aegypti* populations based on macro-geographical sampling (country scale) to explore the history of colonization and the current population dynamics of the species in Bolivia. We assessed *Ae. aegypti* genetic diversity and genetic structure by using both nuclear (microsatellite) and mtDNA markers.

2. Materials and methods

2.1. Study sites and mosquito sampling

Ae. aegypti mosquitoes were sampled between November 2005 and November 2007 as preimaginal stages in 21 localities covering various habitats of Bolivia (Fig. 1, Table 1), except in Caiza, Cobija and El Palmar, where some adult specimens included in phylogenetic studies were collected as adults with nets. Each sample consisted of larvae or pupae collected from multiple breeding sites in each location. Larvae and pupae were reared until the adult stage for morphological identification, and the adults were then stored at -20°C for molecular analysis.

2.2. Microsatellite Analysis

Only fourteen of the 21 prospected sites exhibited sufficient specimen numbers ($n > 20$) and were included in the microsatellite analysis (see table 1). Total DNA was extracted from whole mosquito bodies, as described by Morlais et al. (2004). DNA pellets were re-suspended in sterile water. Genetic polymorphism was assessed at nine microsatellite markers (3472, 3838, AEDGA, AED19, AEDC, T3A7, A10, H08, M313). The microsatellites and PCR conditions have previously been detailed (Paupy et al., 2008, 2010). DNA was amplified in a GeneAmp 9600 thermal cycler (PerkinElmer, USA). PCR products were analyzed using ABI Prism™ 3100 (Applied Biosystems), and genotyping GeneMapper (Applied Biosystems) (for details see Paupy et al., 2008).

The presence and frequencies of null alleles at each locus were checked within each population by using MICROCHECKER V2.2.3 (Van Oosterhout et al., 2004). Genetic diversity per locus and per sample was characterized by the number of alleles and the estimated allele richness, R_s (El Mousadik and Petit, 1996), using

Table 2
Microsatellite genetic variability loci and Hardy–Weinberg tests in populations surveyed in Bolivia.

Locality (N)	34/72			A10			AEDGA			T3A7			38/38			AED19			AEDC			H08			M313			All loci		
	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}	N _{alt}	R _s	F _{IS}
Cobija (51)	3	2.51	-0.04	5	3.82	0.12	2	1.87	-0.04	3	3.00	0.00	2	1.41	-	4	3.21	0.00	2	1.92	-0.06	4	3.40	0.06	6	4.89	0.49	30	2.90	0.01
Trinidad (45)	1	1.00	-	3	2.86	0.32	1	1.00	-	3	2.92	0.18	2	1.89	-0.03	2	1.35	-	2	1.36	-	2	2.00	0.30	2	2.00	0.77	18	1.82	0.30
Alcoche (24)	2	1.71	-	2	1.99	-0.08	1	1.00	-	2	2.00	-0.10	1	1.00	-	1	1.00	-	1	1.00	-	2	2.00	0.90	3	2.95	0.56	15	1.63	0.12
Caranavi (45)	3	2.38	0.05	4	3.29	0.80	1	1.00	-	2	2.00	0.33	2	1.472	-	4	2.82	-0.06	1	1.00	-	4	2.81	0.08	4	3.65	0.67	25	2.27	0.35
Santa Fe (20)	1	1.00	-	3	2.94	0.66	1	1.00	-	2	2.00	0.23	1	1.00	-	2	2.00	-0.06	2	2.00	-0.06	2	2.00	0.41	2	2.00	0.22	16	1.78	0.27
Villa Tunari (34)	2	1.99	-0.14	4	3.85	0.20	1	1.00	-	3	2.77	0.38	2	2.00	-0.18	4	3.58	-0.05	2	1.85	-0.02	2	2.00	0.24	4	3.85	0.38	24	2.54	0.15
Montero (45)	2	1.99	-0.10	4	2.93	0.74	1	1.00	-	3	2.77	0.10	2	1.39	-	2	1.78	-0.02	2	1.94	0.14	3	2.95	0.01	3	2.99	0.45	22	2.23	0.10
Santa Cruz de la Sierra (70)	2	1.96	-0.08	5	3.40	0.05	2	1.25	-	3	2.77	0.23	2	1.97	-0.06	2	1.91	-0.05	2	1.99	0.14	2	2.00	0.00	4	3.99	0.65	24	2.36	0.25
La Guardia (41)	2	1.99	-0.13	5	4.49	0.40	1	1.00	-	3	2.83	0.15	2	1.94	-0.04	2	1.83	-0.03	2	2.00	0.02	2	2.00	0.19	5	4.44	0.69	24	2.50	0.20
Robore (38)	2	1.51	-	5	3.70	-0.10	2	1.77	-0.02	3	3.00	0.16	2	2.00	-	3	2.57	-0.01	2	1.75	1.00	2	2.00	-0.16	3	2.61	0.82	24	2.32	0.23
Puerto Suarez (44)	3	2.05	-0.02	3	2.26	-0.03	2	1.66	-0.01	3	2.95	0.16	1	1.00	-	3	2.29	-0.03	2	1.93	-0.05	4	3.20	0.09	4	3.79	0.30	25	2.65	0.10
Camiri (49)	3	2.34	-0.04	6	4.68	0.14	2	1.82	-0.03	2	2.00	0.23	2	1.91	-0.04	2	1.72	-0.02	2	1.95	-0.07	3	2.82	-0.04	5	4.30	0.39	27	2.62	0.13
Caiza (40)	2	2.00	-0.19	6	5.37	0.55	1	1.00	-	4	3.79	0.29	2	2.00	-0.09	4	3.91	-0.09	2	1.85	-0.03	3	2.83	-0.22	3	2.84	0.45	27	2.84	0.13
Yaculba (44)	3	2.38	-0.06	7	4.97	0.00	2	1.79	-0.02	4	3.51	0.37	2	1.87	-0.07	3	2.97	-0.07	2	1.80	-0.02	5	3.59	-0.08	4	3.61	0.12	32	2.94	0.09
Rural (x3, 84)	2	1.99	-0.18	6	4.60	0.47	1	1.00	-	4	3.34	0.18	2	1.96	-0.23	4	3.67	-0.09	2	1.77	-0.04	3	2.42	-0.10	3	2.87	0.42	27	2.62	0.15
Urban (x11, 506)	4	2.22	-0.01	9	4.07	0.19	3	1.47	-0.03	4	3.34	0.20	3	1.91	-0.08	6	2.73	-0.04	2	1.88	0.07	6	2.90	0.06	6	4.26	0.51	43	2.71	0.18
Dry Forest (x8, 371)	4	2.17	-0.01	9	4.40	0.18	3	1.47	-0.02	4	3.33	0.22	3	1.92	-0.11	5	2.80	-0.06	2	1.92	0.08	5	2.82	0.01	5	4.14	0.47	40	2.77	0.15
Moist Forest (x6, 219)	3	2.14	0.14	7	3.97	0.31	2	1.33	-0.04	4	2.98	0.17	3	1.96	-0.12	6	3.10	-0.04	2	1.65	-0.05	5	2.81	0.16	6	4.11	0.54	36	2.67	0.21
All Samples (x14, 590)	4	2.19	-0.06	9	4.24	0.23	3	1.41	0.27	4	3.20	0.2	3	1.94	-0.11	6	2.99	-0.05	2	1.86	0.06	6	2.85	0.06	6	4.14	0.49	43	2.76	0.17

(N): Sample sizes (the number preceded by "x" indicates the number of samples concerned with the regrouping); N_{alt}: number of scored alleles; R_s: Allele richness (El Mousadik and Petit, 1996); F_{IS}: Inbreeding coefficient, calculated according to (Weir and Cockerham, 1984); Boldfedi: significant deficit in heterozygotes (P < 0.05) after correction using the Bonferroni procedure (Holm, 1979).

FSTAT2.9.3.2 (Goudet, 1995). The linkage disequilibrium between pairs of loci, deviations from Hardy–Weinberg equilibrium (HWE), and the genetic differentiation were estimated with GENEPOP 4.07 (Raymond and Rousset, 1995). F_{IS} and F_{ST} estimates were calculated as described in Weir and Cockerham (1984), and tested for statistical significance with exact tests available in GENEPOP 4.07. The overall significance of multiple tests was estimated with Fisher's combined probability test. Critical significance levels for multiple testing were corrected with the sequential Bonferroni procedure (Holm, 1979). Isolation by distance (IBD) was estimated with Mantel's test in GENEPOP 4.0.7, using the correlation between genetic and geographic distances by the regression of pairwise F_{ST}/(1 - F_{ST}) on the natural logarithm (Ln) of straight-line geographic distance (Rousset, 1997). Neighbor-joining trees were computed using POWERMARKER 3.25 (Liu and Muse, 2005) based on the genetic distances among populations. The robustness of trees was evaluated by bootstrapping over loci. One thousand bootstrapped trees were summarized to obtain a consensus tree, using the CONSENSE module in the Phylip package (Felsenstein, 1989), followed by editing with TREEVIEW (Page, 1996).

2.3. MtDNA polymorphism

The polymorphism in the mitochondrial genes ND4 and COI was explored by using, according to the site, 1–15 *Ae. aegypti* specimens from 20 of the 21 sampled Bolivian localities (see Table 1 and 5). DNA of Bolivian and extra-bolivian (i.e. individuals sampled worldwide outside Bolivia; see Supplemental Table S1) mosquitoes was extracted using the method described previously. A 400 bp fragment of ND4 was amplified using previously published primers FOR-ND4 and REV-ND4 (Da Costa-da-Silva et al., 2005). Concerning COI, a primer pair, 5'-TGTAATTGTAACAGCTCATGCA-3' (COI-FOR)/5'-AATGATCATAGAAGGGCTGGAC-3' (COI-REV) amplifying a 860 bp fragment was designed using the *Ae. aegypti* COI sequence (AF390098 and cover positions 165–1025). PCR amplification was performed using a GeneAmp 9600 thermal cycler (PerkinElmer) with 50 µl reaction mixes containing 4 µl of 1/10 template DNA dilution, 5 µl of 10× reaction buffer (Qiagen), 0.2 mM dNTP, 20 pmol of each primer and 1 U of Taq polymerase (Qiagen, USA). The thermal cycling conditions consisted of 2 min at 94 °C, 35 cycles of 1 min at 94 °C, 30 s at TA (56 °C for ND4 and 54 °C for COI), and 1 min at 72 °C, followed by 10 min at 72 °C. PCR products were detected by agarose gel electrophoresis in Tris–borate-EDTA buffer (TBE), stained with 0.5 µg/ml ethidium bromide in TBE, and visualized under UV light. PCR products were purified with the Ampure kit (Beckman Coulter, France, Paris) and sequenced on both strands using ABI Prism BigDye Terminator version 3.1 (Applied Biosystems). Each 10 µl sequencing reaction mix contained 1 µl of Ready Reaction mix (Applied Biosystems), 1 µl of 5× sequencing buffer, 3.2 pmol/µl primer, and 0.5–1.0 µl of purified PCR product. The reaction consisted of an initial denaturation step of 1 min at 96 °C, followed by 25 cycles of 10 s at 96 °C, 5 s at 50 °C, and 4 min at 60 °C, with a final elongation step of 3 min at 72 °C. Sequencing reaction products were purified with the Agencourt CleanSeq kit (Beckman Coulter) and analyzed on an ABI 3130XL automatic sequencer (Applied Biosystem).

Sequences were analyzed using SEQSCAPE software 2.5 (Applied Biosystems), aligned with Clustal W (Thompson et al., 1994), and numbered in reference to the published *Ae. aegypti* complete sequences of COI (bases 1–1537, Genebank accession No. AF390098) and ND4 (bases 1–1344, GeneBank accession No. DQ440274). Basic sequence statistics including haplotype frequency, nucleotide diversity (π) and neutrality tests of Tajima

(1989), Fu and Li (1993), and Fu (1997) were computed in DNASP 4.10.9. (Rozas et al., 2003).

Median-joining haplotype networks (Bandelt et al., 1999) based on COI and ND4 were drawn using NETWORK 4.6 (Fluxus Technology Ltd 1999–2010, 1999) to infer the relationships between haplotypes. The phylogenetic relationships between COI and ND4 haplotypes recorded in and outside Bolivia were additionally explored with Bayesian inference analysis. MRMODELTEST v2.2 (Nylander, 2004) was used to select the model best fitting the COI and ND4 nucleotide sequence data (under Akaike's information criterion). We carried out a COI–ND4 combined analysis to enhance phylogenetic information with MRBAYES 3.1.2 (Ronquist and Huelsenbeck, 2003) and four Markov chains were run for 2000,000 generations (sampling every 100 generations) to allow adequate time for convergence. The first 2500 trees were discarded as burn-in, and the remaining 17,500 sampled trees were used to estimate the 50% majority rule consensus tree and the Bayesian posterior probabilities. All MCMC runs were repeated twice to confirm consistent approximation of the posterior parameter distributions. The resulting tree was edited in TREEVIEW (Page, 1996).

3. Results

3.1. Microsatellite analysis

3.1.1. Genetic variability

Genotypes at nine microsatellite loci were determined for 590 *Ae. aegypti* specimens collected in 14 Bolivian locations (Table 2). All loci were polymorphic, showing a number of distinct alleles ranging from two (AEDC) to nine (A10). Across all loci, the total number of alleles detected and the average allelic richness (R_s) ranged from 15 (Alcoche) to 32 (Yacuiba) and from 1.629 (Alcoche) to 2.945 (Yacuiba), respectively. Over all loci, the genetic diversity assessed using microsatellites appeared higher in southern localities close to the border between Bolivia and Argentina. After grouping the samples according to their environment (i.e. rural vs urban or dry vs moist forest) the R_s index remained comparable between groups.

Departures from Hardy–Weinberg proportions were associated with significant heterozygote deficits in 10 (2 and 8 concerning, respectively A10 and M313 loci) out of a 101 possible tests, across all loci and collections. The presence of null allele was suspected at locus M313 (in 12 of 14 samples), and it was therefore excluded from the dataset for genetic structure analysis. No significant linkage disequilibrium was found among the 376 possible tests.

3.1.2. Genetic differentiation

Overall genetic differentiation between samples was high and statistically significant ($F_{ST} = 0.0627$; $P < 0.0001$). Pairwise estimates of F_{ST} (θ_{ij}) calculated over all loci (Table 3) revealed significant genetic differentiation for 63 of the 91 sample pairs tested, suggesting that the Bolivian populations of *Ae. aegypti* were strongly genetically structured. No statistically significant correlation was detected between genetic distance (estimated as $F_{ST}/(1 - F_{ST})$) and geographic distance between populations (Mantel test, $P > 0.5$). This genetic distance (Nei, 1983) was used to construct the NJ tree (Fig. 2), showing that the genetic structure and the relationship between samples were not related to geographical distance, habitat (i.e. urban vs rural) or bioclimatic environment.

3.2. MtDNA analysis

3.2.1. COI gene

Sequences of the mtDNA COI gene were retrieved from 127 specimens originating from 20 Bolivian localities. Complete overlap of

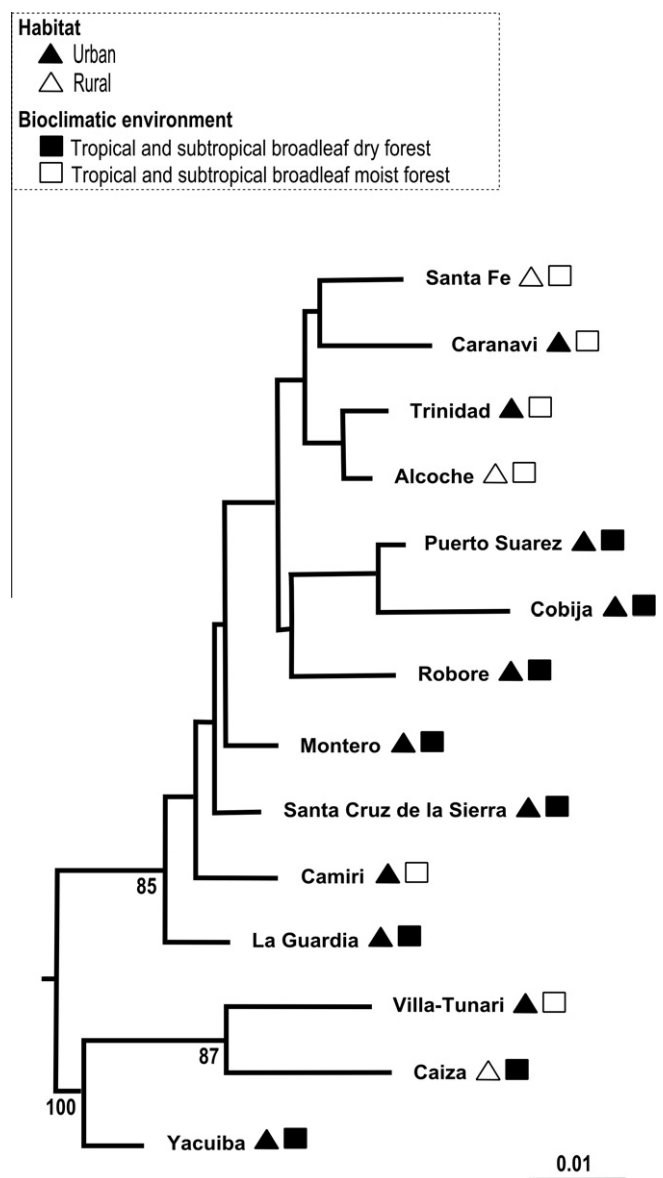


Fig. 2. Dendrogram based on microsatellite Nei's genetic distance (Nei, 1983) clustering by the NJ method. The genetic relationships among the 14 *Ae. aegypti* populations sampled in Bolivia and the habitat and bioclimatic environment of each sample are shown.

all fragments spanned 764 nucleotides, of which 19 were polymorphic (overall nucleotide diversity, $\pi = 0.00135$), defining 8 distinct haplotypes (haplotypes i–VIII; Table 4). Haplotype I was dominant over all sampled localities (frequency = 0.91, Table 5), whereas the seven remaining haplotypes had frequencies of less than 0.03 and were more frequent in rural areas. Haplotype I was detected in two specimens from La Martinique Island and French Guiana. As represented in Fig. 3, the relationships between COI haplotypes suggested the existence of two distinct genetic lineages separated by 11 mutational steps. The first lineage (Haplotypes I to VI) was dominant and found all over Bolivia, whereas the second (Haplotypes VII and VIII) was restricted to the extreme south of the country in rural localities of Caiza and Puente Barrial (Table 5). Considering lineage 1 ($n = 123$), the significant departure from neutrality, as determined by Tajima's (1989) D statistic found at the COI locus ($D = -2.47$, $P < 0.01$), suggested an excess of rare alleles that could reflect demographic instability (population expansion) and/or purifying selection. Fu and Li's statistics were negative but non significant

Table 3
Matrix of pairwise estimates of microsatellite-based F_{ST} (θ_{ij}) across *Ae. Aegypti* populations and ecotopes in Bolivia.

	Cobija	Trinidad	Alcoche	Caranavi	Santa Fe	Villa Tunari	Montero	Santa Cruz	La Guardia	Robore	Puerto Suarez	Camiri	Caiza	Yacuiba
Cobija	–													
Trinidad	0.107 ^{†††}	–												
Alcoche	0.118 ^{†††}	–0.012	–											
Caranavi	0.127 ^{†††}	0.024 ^{††}	0.004	–										
Santa Fe	0.109 ^{†††}	–0.013	–0.005	0.027	–									
Villa Tunari	0.131 ^{†††}	0.127 ^{†††}	0.149 ^{†††}	0.173 ^{†††}	0.120 ^{†††}	–								
Montero	0.054 ^{†††}	0.037 ^{†††}	0.048 ^{††}	0.084 ^{†††}	0.023	0.090 ^{†††}	–							
Santa Cruz de la Sierra	0.096 ^{†††}	–0.001	–0.002	0.019 ^{†††}	–0.005	0.100 ^{†††}	0.021	–						
La Guardia	0.098 ^{†††}	0.018 ^{††}	0.024	0.046 ^{†††}	0.011	0.067 ^{†††}	0.019	0.004	–					
Robore	0.085 ^{†††}	0.004	0.005	0.014 ^{††}	0.013	0.100 ^{†††}	0.043 ^{††}	0.006	0.027 ^{††}	–				
Puerto Suarez	0.085 ^{†††}	0.003	–0.003	0.017 ^{††}	0.001	0.141 ^{†††}	0.028	0.001	0.026 ^{††}	0.001	–			
Camiri	0.135 ^{†††}	0.023 ^{†††}	0.015	0.008	0.022	0.131 ^{†††}	0.064 ^{†††}	0.011 ^{††}	0.016	0.028 ^{†††}	0.026 ^{†††}	–		
Caiza	0.127 ^{†††}	0.144 ^{†††}	0.149 ^{†††}	0.152 ^{†††}	0.113 ^{†††}	0.023 ^{††}	0.095 ^{†††}	0.108 ^{†††}	0.087 ^{†††}	0.104 ^{†††}	0.136 ^{†††}	0.012 ^{†††}	–	
Yacuiba	0.081 ^{†††}	0.081 ^{†††}	0.086 ^{†††}	0.104 ^{†††}	0.052 ^{††}	0.044 ^{†††}	0.019 ^{††}	0.050 ^{†††}	0.039 ^{††}	0.073 ^{†††}	0.072 ^{†††}	0.075 ^{†††}	0.033 ^{††}	–

Statistical significance of F_{ST} estimates was assessed using the Fisher exact test of homogeneity of genotype frequencies (Raymond and Rousset, 1997). Bold: $P < 0.05$; ††: $P < 0.001$; †††: $P < 0.0001$ after correction for multiple tests (Holm, 1979).

($F_* = -5.96$, $P < 0.02$; $D_* = -6.48$, $P < 0.02$). Negative values of F_* and D_* could indicate an excess of recently derived haplotypes and either population expansion or background selection (Fu and Li, 1993). In lineage 2 ($n = 5$), ($D = -1.22$), ($F_* = -1.29$) and ($D_* = -1.22$) statistics were negative but non significant ($P > 0.02$).

3.2.2. ND4. gene

Sequences of the mtDNA ND4 gene were obtained from 124 specimens. Complete overlap of all fragments spanned 335 nucleotides, of which eight were polymorphic (overall nucleotide diversity, $\pi = 0.00159$), defining four distinct haplotypes (Table 4). Among all Bolivian samples, Haplotype I was dominant (frequency = 0.95, Table 5) and encountered in all localities. Haplotypes II–IV were found at low frequencies (<0.03), only in the villages of Caiza and Puente Barrial (Table 5). As shown with COI, two distinct ND4 genetic lineages separated by four mutational steps were supposed to have occurred (Fig. 3). The first ranged across Bolivia, whereas the second was restricted to Caiza and El

Palmar in southern Bolivia. Nucleotide sequences corresponding to the Bolivian Haplotype I were recorded in specimens from several American countries, from North to South, and from West Africa (Table 4). Similarly, correspondences between Haplotype IV and sequences from Central and South America, South-East Asia, French Polynesia and West Africa were also found. No significant departure from neutrality, as determined by Tajima's (1989) D statistic, was found among lineage 1 ($n = 119$; $D = -1.35$, $P > 0.1$) or lineage 2 ($n = 5$; $D = -1.64$, $P > 0.1$). Fu and Li's statistics were negative and significant in lineage 1 ($F_* = -2.84$, $P < 0.05$; $D_* = -1.24$, $P < 0.05$) and non significant in lineage 2 ($F_* = 1.67$, $P > 0.1$; $D_* = 1.64$, $P > 0.05$). It is important to note that the rare COI (VII and VIII) and ND4 (III and IV) haplotypes were always associated together, proving the congruence between the two mtDNA markers.

3.2.3. Combined COI–ND4 analysis

After concatenated sequences with DAMBE (Xia and Xie, 2001), we therefore performed a combined COI–ND4 sequence analysis to

Table 4
MtDNA COI and ND4 haplotypes recorded across *Ae. aegypti* populations from Bolivia.

Haplotype	Frequency	COI	ND4	Reference
		2 2 2 2 2 3 3 3 3 5 5 5 6 6 6 6 8 8		
		0 6 6 7 7 8 3 5 6 9 1 4 8 1 6 7 9 2 8 (1)		(2)
		7 0 3 3 6 5 1 7 9 0 9 0 3 8 9 5 9 8 8		
Haplotype I	117	G C C T C G T A A T G C C A G C T A T	Martinique 2/3	Isolate COI-Guy (French Guiana)
Haplotype II	1 T		
Haplotype III	1	. A		
Haplotype IV	1	. . A		
Haplotype V	1 G		
Haplotype VI	1 A		
Haplotype VII	4	A . . C T A . . G C A . . G A T C . C		
Haplotype VIII	1	A . . C T A C G G C A T . G A T C . C		
		6 7 7 1 8 8 8 9		
		9 5 6 6 1 8 9 3	(1)	(3) (4) (5)
		3 3 2 7 2 8 7 3		
Haplotype I	118	C T C C C G C T	Martinique 2/3	H11 (Brazil)
Haplotype II	1	T C		H6 H7 and H8 (Brazil, (Mexico) Venezuela, USA, Senegal)
Haplotype III	2	T C T T		
Haplotype IV	3	T C T T T A T C	Mexico, 4, Cambodia 1/2/3, Thailand 1/2, Vitenam 1/2, Guinea 1/2, RCI 1/2	H3 (Brazil)
				H5 H13 (Brazil, Cambodia, (Mexico) Singapore, French Polynesia)

Only polymorphic positions are shown, and these are numbered with reference to the published *Ae. aegypti* (Red Eyes Strain) sequence of cytochrome oxidase subunit I, GeneBank accession No. AF390098 and *Ae. aegypti* complete sequence of the NADH dehydrogenase subunit 4, GeneBank accession No. DQ440274. Dots represent identity with respect to reference. The frequency indicates the number of times the haplotype was found in the total sample. Correspondence with haplotypes described in (1) this study, (2) Fort et al. (in press), (3) Lima Júnior and Scarpassa (2009), (4) Gorrochotegui-Escalante et al. (2002), (5) Bracco et al. (2007).

Table 5
Frequencies of MtDNA COI and ND4 haplotypes across *Ae. aegypti* populations and ecotopes in Bolivia.

Locality	COI									ND4				
	N	I	II	III	IV	V	VI	VII	VIII	N	I	II	III	IV
Cobija	7	1.00	–	–	–	–	–	–	–	9	1.00	–	–	–
Porvenir	2	1.00	–	–	–	–	–	–	–	1	1.00	–	–	–
Trinidad	5	1.00	–	–	–	–	–	–	–	6	1.00	–	–	–
Puerto Balivian	2	0.50	–	–	–	–	0.50	–	–	1	1.00	–	–	–
Puerto Almacen	2	0.50	–	0.50	–	–	–	–	–	1	1.00	–	–	–
Caranavi	15	0.86	–	–	0.07	0.07	–	–	–	15	1.00	–	–	–
Alcoche	5	1.00	–	–	–	–	–	–	–	6	1.00	–	–	–
Santa Fe	5	1.00	–	–	–	–	–	–	–	5	1.00	–	–	–
Villa Tunari	6	1.00	–	–	–	–	–	–	–	3	1.00	–	–	–
Chipiriri	2	1.00	–	–	–	–	–	–	–	2	1.00	–	–	–
Montero	6	1.00	–	–	–	–	–	–	–	6	1.00	–	–	–
Santa Cruz de la Sierra	9	1.00	–	–	–	–	–	–	–	9	1.00	–	–	–
Robore	7	1.00	–	–	–	–	–	–	–	6	1.00	–	–	–
Santiago de Chiquitos	3	1.00	–	–	–	–	–	–	–	3	1.00	–	–	–
Puerto Suarez	10	1.00	–	–	–	–	–	–	–	10	1.00	–	–	–
Camiri	10	1.00	–	–	–	–	–	–	–	10	1.00	–	–	–
Yacuiba	10	1.00	–	–	–	–	–	–	–	9	1.00	–	–	–
Puente Barial	5	0.60	–	–	–	–	–	0.40	–	5	0.60	–	0.20	0.20
Caiza	13	0.69	0.08	–	–	–	–	0.15	0.08	14	0.71	0.07	0.07	0.14
El Palmar	3	1.00	–	–	–	–	–	–	–	3	1.00	–	–	–
Urban	85	0.98	–	–	0.01	0.01	–	–	–	83	1.00	–	–	–
Rural	42	0.81	0.02	0.02	–	–	0.02	0.10	0.02	39	0.85	0.02	0.05	0.07
Dry Forest	76	0.92	–	0.02	0.02	0.02	0.02	–	–	73	0.94	0.01	0.02	0.03
Moist Forest	47	0.92	0.01	–	–	–	–	0.05	0.01	49	1.00	–	–	–
Bolivia	127	0.91	0.01	0.01	0.01	0.01	0.01	0.03	0.01	124	0.95	0.01	0.02	0.03

N: number of specimens analyzed; Roman numerals correspond to the name of COI or ND4 haplotypes.

infer the geographic and phylogenetic relationships between Bolivian and extra-bolivian specimens of *Ae. aegypti*. The resulting phylogenetic tree using Bayesian inference (Fig. 4) showed two main groups, which were nevertheless moderately supported by posterior probabilities (i.e. <0.95). As expected, the first group contained the Bolivian specimens with combinations of COI–ND4 haplotypes from Lineage 1, whereas the second included specimens with combinations from Lineage 2. Both lineages were related to extra-bolivian specimens originated from South–East Asia, America and Africa. The lineage 1 included specimens exclusively from East Africa (Tanzania), Central (Mexico) and South (Brazil) America, and the Caribbean region (Martinique). The second lineage also included specimens from Africa but exclusively West Central Africa (Cameroon, Guinea and Republic of Côte d'Ivoire). This group additionally included mosquitoes from North (USA), Central (Mexico) and South America (Venezuela).

4. Discussion

This is the first study of the genetic variation and differentiation of *Ae. aegypti* in Bolivia. The results reveal a significant level of genetic structure at the country scale, and also suggest the existence of two genetic lineages of this species in Bolivia.

4.1. Genetic diversity and genetic structure

In Bolivia, the levels of genetic diversity found with microsatellites across *Ae. aegypti* populations were low compared to those reported in native African populations using the same set of genetic markers (Paupy et al., 2008, 2010). Such a result illustrated the loss of genetic diversity due to founder effects that occurred during the invasion of *Ae. aegypti* in Bolivia. The level of genetic diversity found in Bolivia was lower than those observed in other introduced populations in America. Whereas 10 COI haplotypes were previously detected across 14 Brazilian localities with an overall nucleotide diversity (π) of 0.00651 (Scarpassa et al., 2008), only eight were detected in this study, with a nucleotide diversity about

one-fifth of the former ($\pi = 0.00135$). The ND4 gene also usually provided a higher number of haplotypes (N) and higher nucleotide diversity in South ($N = 14$, $\pi = 0.01997$ in Brazil, Bracco et al., 2007; $N = 7$, $\pi = 0.01877$ in Venezuela, Herrera et al., 2006) and Central America ($N = 25$, $\pi = 0.01636$ and 0.02161 in Mexico, Gorrochotegui-Escalante et al., 2002) than those observed here ($N = 4$, $\pi = 0.00159$). Such a lack of genetic diversity could be explained by the geographic isolation of Bolivia and by the poor terrestrial access (Gozalves et al., 2000), that probably delayed and limited re-invasion of Bolivia by *Ae. aegypti*.

Microsatellite analysis revealed a strong genetic structure among *Ae. aegypti* populations sampled at the macro-geographic scale. Both statistics of Tajima and Fu and Li computed with COI sequences suggested that the *Ae. aegypti* population expanded recently in Bolivia. The founder effect that usually occurs during the expansion phase of invasive species drastically decreased the effective size of founder populations and their genetic diversity. Under genetic drift, established populations tended to diverge, especially when migration rates between them are restricted, as is the case in Bolivia due to the poor road network. The re-expansion of *Ae. aegypti* in other South-American countries such as Brazil and Venezuela also led to a strong level of genetic structure at the country scale (Lourenço-de-Oliveira et al., 2004; Urdaneta-Marquez et al., 2008). The same pattern of genetically differentiated populations has also been observed for other invasive mosquito species, such as *Aedes albopictus* in Cameroon (Kamgang et al., 2011).

4.2. Multiple genetic lineages

In this study, two mtDNA lineages of *Ae. aegypti* were recovered among Bolivian samples, whatever the marker considered (COI or ND4). This finding is in accordance with previous surveys undertaken in Argentina (Rondan Dueñas et al., 2009), Brazil (Bracco et al., 2007), Peru (da Costa-da-Silva et al., 2005) and Venezuela (Herrera et al., 2006), that also highlighted the presence of multiple mtDNA lineages.

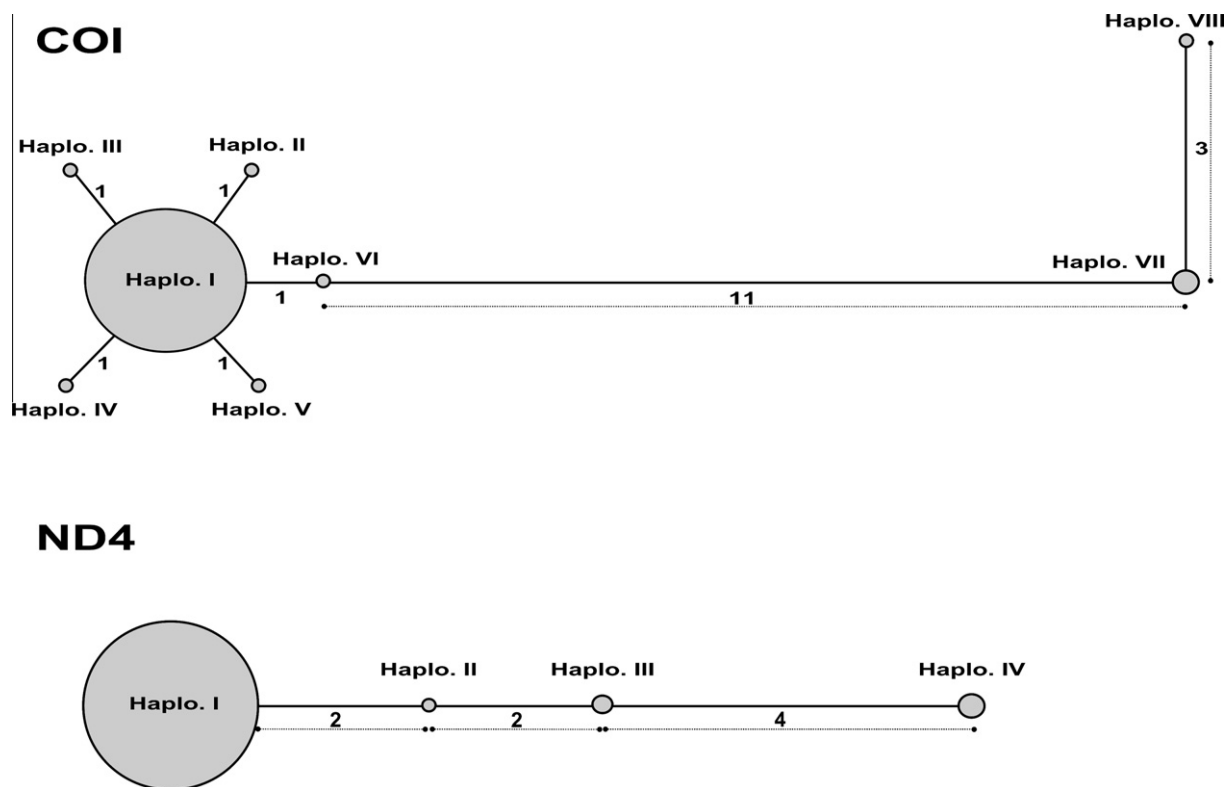


Fig. 3. Median-joining networks based on haplotypes obtained from the mtDNA-COI (in top) and -ND4 (in bottom) sequences as generated by Network 4.6. Haplotypes are represented as ovals, scaled to reflect their frequencies. Lines connecting haplotypes and small circles (missing or non sampled haplotypes) represent a single mutation event. Arabic numbers indicate the number of mutation events.

Several works recently demonstrated that mitochondrial pseudogenes are highly prevalent in the nuclear genome of *Ae. aegypti* (Black and Bernhardt, 2009; Hlaing et al., 2009; Behura et al., 2011). Such a nuclear copies of mtDNA (i.e. Numts), may be PCR amplify in addition, or instead the authentic mtDNA target and generate thus misleading results in population genetic and phylogenetic studies that employed this kind of markers (Hlaing et al., 2009). We wondered how Numts possibly interfered in the present study by generating artefactual mtDNA lineage among mosquitoes sampled, but there are several arguments to believe that we analyzed true mtDNA lineages. First, we used two mtDNA markers (COI and ND4) that both provided congruent results. The rare COI (VII and VIII) and ND4 (III and IV) haplotypes from the lineage 2 were always found associated together, suggesting a physical linkage between them. The majority of Numts are small in length even if one (i.e. supercontig 1.363, see Hlaing et al., 2009) includes COI and ND4 genes (Hlaing et al., 2009). By using a BlastN search in VectorBase against the *Ae. aegypti* whole genome assembly, we compared sequences of Bolivian COI and ND4 haplotypes and the supercontig 1.363. We found 11–16 (COI) and 3–9 (ND4) mismatches between Bolivian haplotypes and the supercontig. Haplotypes of lineage 2 differed by 7–9 and 16 nucleotides for COI and ND4, respectively, suggesting that there was no accurate correspondence between Bolivian haplotypes and the 1.363 Numt. Second, any of our Bolivian PCR products gave ambiguous sequences as it was generally observed when mtDNA and NUMTS were co-amplified. In addition, any nucleotide sequence of Bolivian haplotypes contains End codon, and most of mutations are synonymous. Third, if the lineage 2 had been generated by Numts, then it would have being recovered throughout the Bolivia and not only restricted to southern Bolivia. Moreover, two ND4 haplotypes (Haplotype I and IV, respectively in lineages 1 and 2) corresponded to two

haplotypes previously recovered by Gorrochotegui-Escalante et al. (2002) in Mexico, and that were recently validated as true mtDNA haplotypes and not as Numts (Black and Bernhardt, 2009).

In keeping with the observation made by Scarpassa et al. (2008), the two Bolivian lineages were respectively related to East- and West-African specimens. In Bolivia, lineage 1 is dominant and ranges throughout the country, whereas lineage 2 co-occurs with lineage 1 at a low frequency in rural settings in the extreme south of Bolivia (SB). The presence of two sympatric lineages in SB was corroborated by the higher microsatellite-based genetic diversity indexes we found in Caiza and Puente Barrial. Lineage 1, which presents signatures of recent demographic expansion, probably derives from the colonization wave that started at the beginning of the 1980s. As Bolivia is a landlocked country, it is most likely this wave originated from sources in bordering countries. One of the main entry points to Bolivia is Puerto Suarez in the South–East, via the railway that connects Sao Paulo in Brazil to Santa-Cruz de la Sierra (Gozalves et al., 2000). Thus, it is not surprising that we found strong genetic relationships between some ND4 sequences from Brazilian specimens and the major haplotype of lineage 1. In contrast, the geographical restriction of lineage 2 to the south of Bolivia is suggestive of independent introduction from North-West Argentina (NWA). In this region, where multiple *Ae. aegypti* invasion routes converge, three mtDNA haplogroups were recently detected (Rondan Dueñas et al., 2009; Albrieu Llinás and Gardenal, 2012). Alternatively, lineage 2 could represent a relict lineage that escaped the DDT-based eradication programs supposedly completed in 1943 in Bolivia (Tonn et al., 1982). The low prevalence of this lineage and its ecological restriction to rural settings, that would reflect secondary adaptation, supports this possibility. In La Réunion Island (Indian Ocean), DDT treatment in the 1950s was thought to have led to ecological withdrawal towards wild

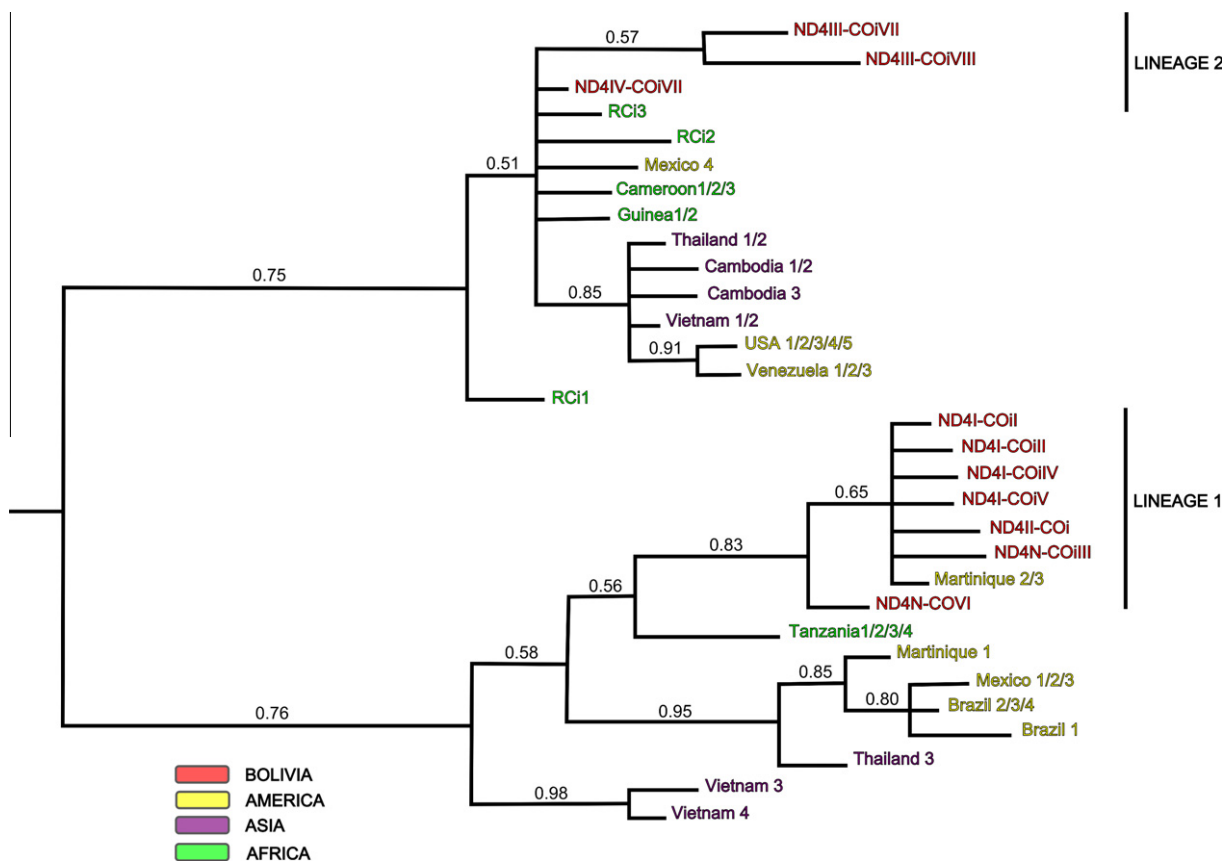


Fig. 4. Bayesian inference hypothesis of *Ae. aegypti* phylogeny based on the combination of COI and ND4 sequence data. Branch support is indicated by the posterior probability values.

habitats surrounding human dwellings (Paupy et al., 2001; Delatte et al., in press). Finally, an additional argument for the “relict lineage hypothesis” is the phylogenetic relation we found between lineage 2 and specimens from West Africa, where the early *Ae. aegypti* introductions in America originated. To confirm this hypothesis, it would be interesting to include in future studies specimens collected prior to 1943 (if available) for comparison with those found today in Bolivia.

It was likely that the American continent was confronted with a population transition during the shift from the pre-eradication era to the post-eradication era in the middle of the 20th century. The impact of such a transition and its potential accompanying phenotypic changes (trophic behaviour, vector competence, etc.) on YF and DEN virus transmission is unknown. Vector competence for both yellow fever and dengue viruses has been linked to the geographic origin of mosquito populations and subspecies (Tabachnick et al., 1985; Failloux et al., 2002). The relation between entomological changes and epidemiological changes observed during the last century, together with the decline of epidemic yellow fever and the emergence of dengue as a major public health threat in South America (Barrett and Monath, 2003), remains more than ever an open question. More efforts are therefore needed to characterize *Ae. aegypti* populations at the biological, genetic and vector-competence levels across South America.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.meegid.2012.04.012>.

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